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Influence of Estrogen, Bevacizumab and Other Factors on VEGF Expression in SKBR-3 Breast Cancer Cells

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Abstract: OBJECTIVE: To investigate expression of VEGF of SKBR-3 breast cancer cells in different estrogen and to investigate the expression of VEGF after Bevacizumab, Trastuzumab, Taxol and Insulin-like growth factor 1 receptor (IGF-1R)antibodies treatment in different estrogen. METHODS: RT-PCR was used to detect the expression of VEGF in SKBR-3 breast cancer cells under different concentrations of estrogen and VEGF monoclonal antibody, HER-2 monoclonal antibody, paclitaxel and IGF-1R antibody. At the same time, ELISA was used to detect the concentration of VEGF protein in the culture supernatant. RESULTS: The expression of VEGF in no estrogen group was 1.0618±0.0085 (VEGF/GAPDH); the expression of VEGF in low concentration group (50pg/L) was 1.0047±0.0061, the difference was statistically significant compared with the no estrogen group (P=0.002); the expression of VEGF in high concentration group (0.2 μg/mL) was 1.0868±0.0135, the difference was statistically significant compared with the no estrogen group (P<0.001). The expression of VEGF were 1.0496±0.0288 and 1.0618±0.0085 in Taxol group and contral Group (F=0.058), the expression of VEGF were 1.0884±0.0036, 1.0618 ±0.0085 in antibody of IGF-1R group and contral group (F=0.073), the expression of VEGF were 0.9887±0.0037, 1.0618±0.0085 in Trastuzumab group and contral group (F=0.075). The expression of VEGF in the paclitaxel group and the control group were 1.0496±0.0288 and 1.0618±0.0085 (F=0.058); The expression of VEGF in IGF-1R group and control group were 1.0884±0.0036 and 1.0618±0.0085(F=0.073); The expression of VEGF in the trastuzumab group and the control group were 0.9887±0.0037 and 1.0618±0.0085(F=0.075); There was no significant change in the expression of VEGF in the paclitaxel group, the IGF-1R group antibody and the trastuzumab group with estrogen. The expression of VEGF in the bevacizumab group and the control group were 1.0057±0.0043 and 1.0618±0.0085(F=0.132, P=0.04), and There is no synergistic effect of estrogen and VEGF monoclonal antibody on the expression of VEGF. Conclusion: The influence of different estrogen on VEGF-expression in SKBR-3 breast cancer cells is remarkable. Taxol, antibody of IGF-1R and Trastuzumab do not change the expression of VEGF in SKBR-3 breast cancer cells, and this has no synergistic effect with estrogen.. Bevacizumab increases the expression of VEGF, and this also has no synergistic effect with estrogen.

Keywords: SKBR-3 Breast Cancer Cell Line, Estrogen, Bevacizumab, Vascular Endothelial Growth Factor, IGF-1R Antibody, Trastuzumab

1. Introduction

The vascular endothelium growth factor (VEGF) plays a key role in tumor angiogenesis. It is highly expressed in many tumor cells and up-regulates the expression of receptors in adjacent vascular endothelial cells to inhibit tumor growth [1]. The expression level of VEGF in cells is correlated with breast cancer. The detection of VEGF can be used as a reference for the diagnosis of breast cancer [2]. VEGF may be associated with a variety of growth factors, which affect the role of other factors through paracrine or autocrine. At the same time, different age of breast cancer patients have different concentrations of estrogen. In SKBR-3 breast cancer cell, different estrogen concentrations affected the effects of bevacizumab (VEGF McAb), trastuzumab (HER-2 McAb), paclitaxel, and insulin-like growth factor 1 recipient (IGF-1R)

on VEGF expression.

2. Materials and Methods

2.1. Materials

Cell culture medium MEM (containing 10% fetal bovine serum and secondary antibody) was purchased from Hy-clone. The RNA extraction kit was purchased from MRC. The cDNA reversal recording kit and the PCRKit reagent kit were purchased from Fermentas. Paclitaxel (batch 100905) and estrogen purchased from Haikou Pharmaceutical Factory Co, Ltd. IGF-1R mAb, HER-2 mAb and VEGF monoclonal antibody were purchased from RnD Corporation. The ELISA kit was purchased from RnD company.

2.2. Cell Lines and Culture Conditions

The breast cancer cell line SKBR-3 was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. All the breast cancer cell s were grown in a 37°C incubator supplied with 5% CO_2 and 95% air.

2.3. Cell Experiment

The experiment was divided into no estrogen group, low concentration estrogen group (0.05μg/mL) and high concentration estrogen group (0.2μg/mL). Each group was divided into with blank group and HER-2 monoclonal antibody group (0.04μg/mL), VEGF monoclonal antibody group (0.5μg/mL), IGF-1R antibody group (2.5μg/mL), and paclitaxel group (1.5μg/mL). And the experiment was repeated 3 times. First, the well-growing cells were taken into the culture bottle (the same number of cells and the culture solution). After 24 hours of culture, the serum-containing medium was removed and the serum-free medium was added. Then, different experimental reagents were added according to the above groups, and the cells or the culture supernatant were collected after 24 hours of culture.

2.4. RT-PCR

First, add 0.5 mL of Trizol to the SKBR-3 breast cancer cells and allow to stand at room temperature for 5 min. Add 0.1 mL of chloroform, mix and allow to stand at room temperature for 5 min, and then centrifuge for 10 min (5000 r/min, r = 8 cm). Then take the upper water sample, add 0.25 mL of isopropyl alcohol, and allow to stand for 10 min at room temperature, and then centrifuge for 10 min (10000 r/min, r=8 cm). Discarde the supernatant, add 0.5 mL of 75% ethanol, mix and centrifuge for 5 min (10000 r/min, r=8 cm), then discarde the supernatant, dry at room temperature, and finally add 20 μL of DEPC treated water and 1 μL of RNA protectant. Take 5 μL of RNA template and add 1 μL of random primer and 6 μL of DEPC treated water for 5 min at 65°C. Add 5×Buffer 4 μL, RNA inhibitor 1 μL, dNTP 2 μL and reverse transcription enzyme 1 μL. After they are mixed, they are stored at 42°C for 60 min, 70°C for 5 min, and -20°C. Amplification conditions: 50°C for 2 min, 95°C for 15 min. Enter the circulation conditions: 94°C 15s, 55°C 40s, 72°C 40s, a total of 40 times. The fluorescence recording temperature was 55°C. The sequence and product size of each primer are shown in Table 1.

Table 1. *IGF-1R, VEGF, HER-2 and GAPDH primer sequences.*

Primer	Upstream	Downstream	Base pair
IGF-1R	CCTCATCCACGCCACAGGCG	TTGGCCTCACAGGGCCACCT	222
VEGF	CACTCAGATCCTGACAGGGAAGA	GCTGGGTTTGTCGGTGTTCCC	162
HER-2	CACTGCCAACCGGCCAGAGG	GACACTCAGGGTGGCACGGC	217
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG	495

2.5. Detection of VEGF Protein Concentration

The concentration of VEGF protein in the supernatant of the culture supernatant was measured by ELISA. Collecte the supernatant of the culture solution and centrifuged for 15 min (3000 r/min, r = 8.5 cm), and then store in a low temperature refrigerator at -80°C. Proceede with the VEGF detection quantification kit according to the instructions in the manual. Make a standard curve and calculated the VEGF protein content in the culture supernatant based on the standard curve.

2.6. Statistical Method

The experimental data were statistically processed using SPSS 17.0. Analysis of variance was used for comparison between groups. After the difference was statistically significant, the LSD method was used for comparison between groups. The test level is α=0.05.

3. Result

3.1. Expression of VEGF

As shown in Figure 1, the expression of VEGF in no estrogen group was 1.0618±0.0085 (VEGF/GAPDH), the expression of VEGF in low concentration group was 1.0047±0.0061 (P=0.002), the expression of VEGF in high concentration group was 1.0868±0.0135 (P<0.001). After analysis of variance, the difference was statistically significant (F = 0.4). Compared with no estrogen group, the low concentration of estrogen increased the expression of VEGF, the difference was statistically significant (P=0.002); Compared with low concentrations of estrogen, high concentrations of estrogen reduced the expression of VEGF, the difference was statistically significant (P < 0.001).

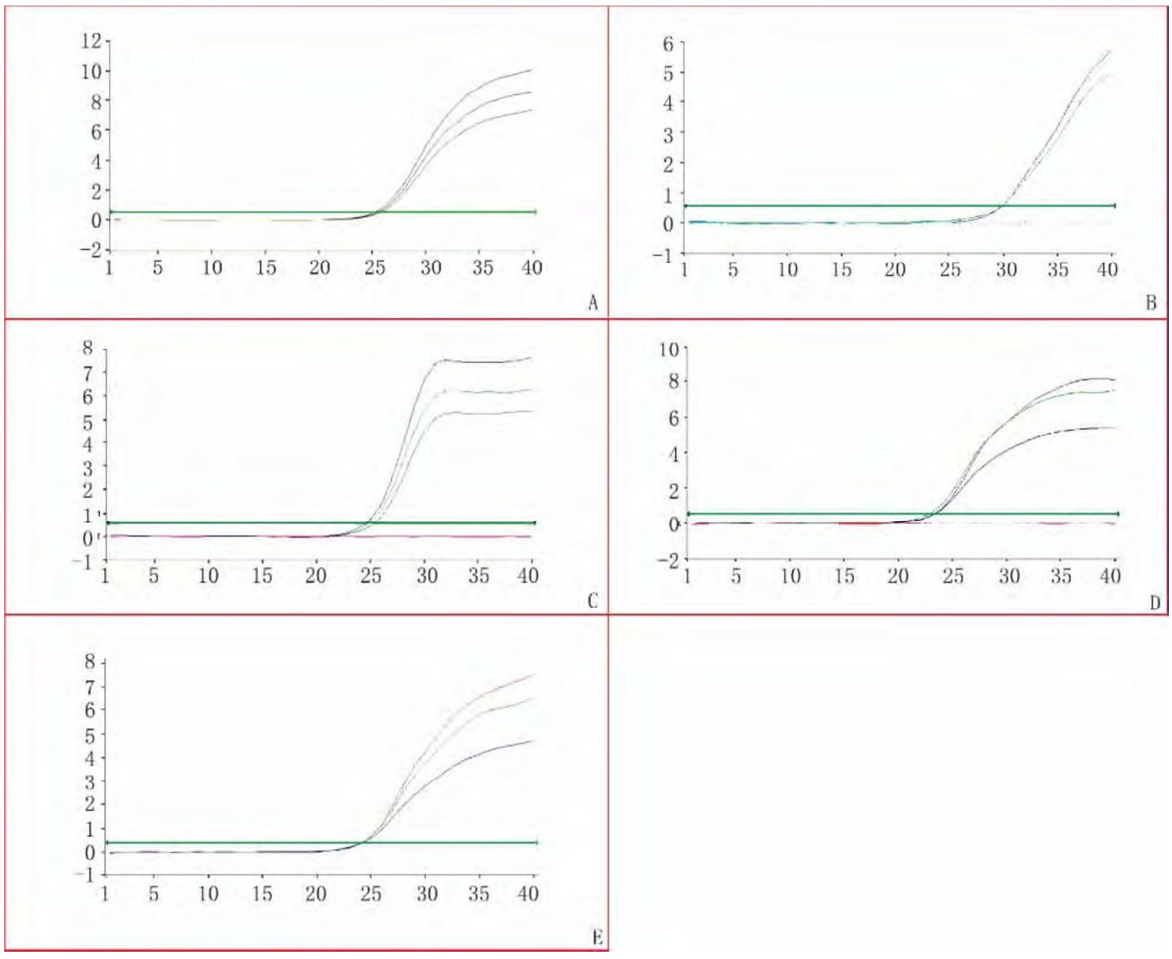
After treatment with paclitaxel, the VEGF expression of SKBR-3 breast cancer cells was 1.0496±0.0288, the low concentration estrogen group (with the paclitaxel)was 1.0433±0.0049, and the high concentration estrogen group

(with the paclitaxel) was 1.0713±0.0069. There was no statistically significant difference compared with estrogen alone (F = 0.058). After the addition of paclitaxel, estrogen increased the expression of VEGF in the presence of low concentrations of estrogen, but the difference was not statistically significant.

The expression of VEGF in breast cancer cells treated with IGF-1R antibody was 1.0884±0.0036, the low concentration estrogen group (with the IGF-1R antibody) was 0.9545±0.0106, and the high concentration estrogen group (with the IGF-1R antibody) was 1.0712. ±0.004. Compared with estrogen alone, the difference was not statistically significant (F=0.073). In the absence of estrogen, the addition of the IGF-1R antibody reduced the expression of VEGF, but the difference was not statistically significant.

The expression of VEGF in breast cancer cells treated with HER-2 antibody was 0.9887±0.0037, the low concentration estrogen group (with the HER-2 antibody) was 1.0231±0.0113, and the high concentration estrogen group (with the HER-2 antibody) 1.0815±0.0037. Compared with the estrogen alone, the difference has no statistical significance (F=0.075). Different from the addition of HER-2 monoclonal antibody, HER-2 monoclonal antibody increased VEGF expression in the absence of estrogen; In the case of low concentrations of estrogen, HER-2 monoclonal antibody decreased VEGF expression, but the difference was not statistically significant.

The expression of VEGF in SKBR-3 breast cancer cells treated with VEGF monoclonal antibody was 1.005±0.0043, the low concentration estrogen group (with the VEGF antibody) was 1.0185±0.006, and the high concentration estrogen group (with the VEGF antibody) was 1.0751± 0.0098. The difference was statistically significant compared with estrogen alone (F = 0.132). Compared with the blank group, VEGF monoclonal antibody increased VEGF expression, the difference was statistically significant (P=0.004); Compared with the VEGF monoclonal antibody group, the expression of VEGF in the low concentration estrogen group (with the VEGF antibody) was decreased, the difference was not statistically significant (P=0.435); Compared with the VEGF monoclonal antibody group, the VEGF expression of the high concentration estrogen group (with the VEGF antibody) was decreased, the difference was statistically significant (P = 0.001); Compared with the low concentration estrogen group (with the VEGF antibody), the expression of VEGF was decreased in the high concentration estrogen group (with the VEGF antibody), the difference was statistically significant (P=0.004).



Note: A. Estrogen; B. Paclitaxel; C. IGF-1R antibody; D. HER-2 antibody; E. VEGF antibody

Figure 1. *Effect of estrogen, paclitaxel, IGF-1R antibody, HER-2 antibody and VEGF antibody on the expression of VEGF in breast cancer cells.*

3.2. Effect on VEGF Protein in Supernatant

In the absence of estrogen, the VEGF protein content in the supernatant was 0.74±0.009; The paclitaxel group was 0.783±0.114; The IGF-1R antibody group was 0.746±0.063; The HER-2 antibody group was 0.953 ± 0.068; The VEGF antibody group was 0.0265 ± 0.001. After analysis of variance, the difference was not statistically significant. VEGF monoclonal antibody significantly reduced the supernatant VEGF protein content; The HER-2 monoclonal antibody increased the VEGF protein content in the supernatant, which was consistent with the elevated VEGF expression of the antibody, but the difference was not statistically significant.

At low concentrations of estrogen, the VEGF protein content of the supernatant was 0.7455 ± 0.023; The paclitaxel group was 0.6852±0.046; The IGF-1R antibody group was 0.86±0.152; The HER-2 antibody group was 0.7553 ± 0.001; The VEGF antibody group was 0.0468 ± 0.005. After analysis of variance, the difference has no statistical significance. The low concentration of estrogen increased the VEGF protein content in the supernatant, which was consistent with the increase in VEGF expression; Paclitaxel inhibited the increase of VEGF protein content in the supernatant of low concentration estrogen and inhibited the expression of VEGF. VEGF monoclonal antibody significantly reduced the VEGF protein content in the supernatant, but not related to estrogen concentration; The HER-2 mAb reduced the VEGF protein content in the supernatant, which was consistent with the decrease in VEGF expression of the antibody, but the difference was not statistically significant.

When the concentration of estrogen is high, the VEGF protein content of the supernatant is 0.446±0.044; The paclitaxel group was 0.308 ± 0.00; The IGF-1R antibody group was 0.5945±0.065; The HER-2 antibody group was 0.5065±0.052; The VEGF antibody group was 0.0175 ± 0.001. After analysis of variance, the difference has no statistical significance. High concentrations of estrogen reduced the level of VEGF protein in the supernatant and the results of decreasing VEGF expression; VEGF monoclonal antibody significantly reduced the VEGF protein content in the supernatant, regardless of estrogen concentration. The increase in VEGF protein content in the supernatant of HER-2 monoclonal antibody was consistent with the increase in VEGF expression.

4. Discussion

Different estrogen levels affect the treatment and prognosis of breast cancer, and the estrogen levels are significantly different before and after menopause. Estrogen can increase the expression of VEGF mRNA when cultured human venous smooth muscle cells in vitro [3]; When they studied the effect of estrogen on the ovarian cancer cell line SKOV3, it was found to increase the level of VEGF mRNA and protein [4]; In estrogen receptor-positive breast cancer cells, estrogen upregulates the expression of VEGF protein and mRNA [5], which depends on the presence of estrogen receptors. In estrogen receptor-negative breast cancer cells, estrogen can also up-regulate the expression of VEGF protein and mRNA, but this may be mediated by an estrogen receptor variant located in the nucleus [6]. Estrogen has an important regulatory effect on angiogenesis in breast cancer tissues. Some related experiments show that this effect is produced by increasing VEGF mRNA expression and promoting VEGF protein secretion. At the same time, it requires the presence of estrogen receptors [7-8]. In MCF-7 breast cancer cells, estrogen increases VEGF transcriptional activity [9]. In this study, low concentrations of estrogen increased VEGF expression in SKBR-3 breast cancer cells, while high concentrations of estrogen decreased VEGF expression. The VEGF protein content in the supernatant was also slightly elevated in the case of low concentrations of estrogen, and slightly decreased in the high concentration. At present, the mechanism of the effect of different estrogen concentrations on the expression of VEGF is unknown. Estrogen and its signaling pathway play an important role in regulating the expression of VEGF in breast cancer patients. In the clinic, clinicians should consider the menstrual status of different breast cancer patients, especially in the case of low estrogen levels (postmenopausal), clinicians should consider the possible effects of elevated VEGF on treatment, and treat VEGF as a target.

Kristine et al [10] found that transfection of HER-2 gene increased VEGF protein level in breast cancer cell line MDA-MB-435, but this did not change its mRNA level, which may be caused by activation of p70S6K pathway; Gunter et al [11] also found that the activation of HER-2 caused an increase in VEGF expression and protein levels when studying mouse tumor cells; Read et al [12] found in breast cancer cell experiments that estrogen down-regulates the expression of HER-2, and anti-estrogen treatment can reverse this phenomenon. The results showed that after the addition of HER-2 monoclonal antibody, VEGF expression was elevated in the absence of estrogen and decreased in low concentrations of estrogen. This may be related to the following factors: 1) HER-2 monoclonal antibody is used in breast cancer cells, which reduces the number of HER-2 receptors, resulting in inhibition of this signaling pathway and decreased expression of VEGF; 2) The addition of estrogen also further reduced the number of HER-2, which reduced the expression of VEGF, and the effect of decreased HER-2 on the expression of VEGF greatly exceeded the effect of low concentration of estrogen on VEGF expression.

Paclitaxel is a commonly used drug for the treatment of breast cancer. It has achieved good results in clinical treatment, especially for patients with higher risk. The use of paclitaxel in the chemotherapy of breast cancer can improve the overall treatment efficiency of patients and the safety of the drug regimen [13]. Studies have shown that VEGF-A protects breast cancer cells from paclitaxel, while inhibition of VEGF-A enhances paclitaxel activity and reduces the likelihood of metastasis [14]. Wang Qing et al [15] found that

the clinical remission rate of docetaxel in patients with premenopausal breast cancer was 66.6%, and 48.1% after menopause. This study found that the effect of estrogen on increasing VEGF expression was inhibited by paclitaxel, and the results were similar to the above studies.

IGF-1R is present on a variety of cell membrane surfaces and it is overexpressed in a variety of tumors. When IGF-1R binds to its ligands (IGF-I and IGF-II), it promotes tumor cell proliferation, promotes malignant transformation of cells, and inhibits tumor cell apoptosis. Therefore, inhibition of IGF-1R can inhibit the growth of experimental tumors [16]. Beckert et al [17] found that IGF-I increases VEGF expression and protein levels in vascular endothelium, thereby promoting vascular growth. The experiment showed that IGF-1R monoclonal antibody reduced VEGF expression in the absence of estrogen, but estrogen exerted a greater effect on VEGF than IGF-1R monoclonal antibody.

HER-2 has a role in regulating cell growth, survival and differentiation. It plays a key role in cell growth factor signaling pathway and in controlling cell growth and division. The monoclonal antibody against HER-2 has achieved good therapeutic effects in clinical applications [18-19]. In the present experiment, HER-2 monoclonal antibody increased the expression of VEGF in the absence of estrogen, and the protein content in the supernatant also showed the same trend; in the low concentration of estrogen, HER-2 monoclonal antibody decreased the expression of VEGF. It has been found that HER-2 monoclonal antibody can decrease the expression of VEGF-C, suggesting that VEGF is a target downstream of the HER-2 signaling pathway [20]. Differences from this experiment may differ from the target, one is VEGF-C, another is VEGF-A, and there is a correlation with the presence of estrogen.

VEGF monoclonal antibody is a neutralizing antibody of VEGF, which has a clear target and can inhibit the growth of blood vessels. Compared with chemotherapy alone, it significantly improves the progression-free survival and remission rate of patients [21], and the risk of progression is reduced by 31% to 52%. Bevacizumab is effective in the treatment of advanced breast cancer and can effectively improve cytokine levels such as VEGF [22]. The adverse reactions are mild and easy to handle. In the experiment, VEGF monoclonal antibody significantly increased the expression of VEGF in cells. This phenomenon is due to the decrease in VEGF protein in the supernatant, which stimulates the increase of mRNA synthesis in the cells. The supernatant VEGF protein decreases after the addition of VEGF monoclonal antibody, and the VEGF monoclonal antibody also counteracts the effect of low concentration of estrogen on VEGF.

5. Conclusion

In conclusion, this study investigated the effects of different estrogen concentrations and/or paclitaxel, IGF-1R monoclonal antibody, HER-2 monoclonal antibody, and VEGF monoclonal antibody on VEGF expression in estrogen receptor-negative and HER-2 positive breast cancer cells. This experimental study found that low concentrations of estrogen increased VEGF expression, while high concentrations of estrogen decreased VEGF expression; At the same time, this study also found that VEGF monoclonal antibody increased the expression of VEGF, and estrogen did not affect the effects of HER-2 monoclonal antibody, VEGF monoclonal antibody, IGF-1R antibody and paclitaxel on VEGF. At present, the expression of VEGF in breast cancer cells is very important. With the advancement and development of medicine, the future of related treatment is very promising and worthy of further exploration.

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